

# A Mutated $\beta$ -Catenin Gene Encodes a Melanoma-specific Antigen Recognized by Tumor Infiltrating Lymphocytes

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## Summary

A number of antigens recognized by tumor-reactive T cells have recently been identified. The antigens identified in mouse model systems appear, with one exception, to represent the products of mutated genes. In contrast, most of the antigens recognized by human tumor-reactive T cells reported to date appear to represent the products of non-mutated genes. Here we report the isolation of a cDNA clone encoding  $\beta$ -catenin, which was shown to be recognized by the tumor-infiltrating lymphocyte (TIL) 1290, a HLA-A24 restricted melanoma-specific CTL line from patient 888. The cDNA clone, which was isolated from the autologous melanoma cDNA library, differed by a single base pair from the published  $\beta$ -catenin sequence, resulting in a change from a serine to a phenylalanine residue at position 37. Normal tissues from this patient did not express the altered sequence, nor did 12 allogeneic melanomas, indicating that this represented a unique mutation in this patient's melanoma. A peptide corresponding to the sequence between amino acids 29 and 37 of the mutant gene product was identified as the T cell epitope recognized by TIL 1290. The observation that HLA-A24 binding peptides contain an aromatic or hydrophobic residue at position 9 suggested that the change at position 37 may have generated a peptide (SYLDSGIHF) which was capable of binding to HLA-A24, and a competitive binding assay confirmed this hypothesis. The  $\beta$ -catenin protein has been shown previously to be involved in cell adhesion mediated through the cadherin family of cell surface adhesion molecules. The high frequency of mutations found in members of cellular adhesion complexes in a variety of cancers suggests that these molecules may play a role in development of the malignant phenotype.

The majority of murine antigens recognized by tumor-reactive T cells represent the products of mutated genes (1, 2). The only reported example of a non-mutated gene product recognized by tumor reactive T cells in the mouse is the P1A antigen of the P815 mastocytoma, which is also expressed on some normal mast cell lines (3). A number of genes encoding antigens recognized by human melanoma-reactive T cells have recently been isolated. The MAGE (4, 5), BAGE (6), and GAGE (7) gene products are non-mutated proteins and are not expressed in normal tissues with the exception of testes, and thus appear to represent tissue-specific antigens with a restricted pattern of expression. In addition, melanoma-reactive T cells have been shown to recognize tyrosinase (8, 9), MART-1 (10), gp100 (11), and TRP-1 (12). Non-mutated epitopes were identified in these proteins, whose expression in normal tissues was limited to melanocytes and retina. Two reports have

recently appeared, however, demonstrating that human melanoma-reactive T cells recognize mutant gene products (13, 14).

The HLA-A24 restricted melanoma-reactive tumor-infiltrating lymphocyte (TIL)<sup>1</sup> 1290 cell line established from patient 888 was recently shown to recognize the product of a newly described gene termed p15 (15). This product appeared to be expressed in a wide variety of normal tissues, and a non-mutated peptide from this protein was recognized by TIL 1290. This report presents the isolation of a second gene encoding an antigen recognized by TIL 1290. Two clones which were isolated by screening a cDNA library with TIL 1290 represented partial cDNAs derived from the  $\beta$ -catenin gene. The T cell epitope identified in

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<sup>1</sup>Abbreviations used in this paper: mel, melanoma; TIL, tumor-infiltrating lymphocytes.

this product was encoded by a region containing a single point mutation which gave rise to a peptide with significantly enhanced binding to the HLA-A24 class I molecule.

$\beta$ -catenin has been shown to be involved in the binding of members of the cadherin family of cell surface adhesion molecules to cytoplasmic actin microfilaments (reviewed in 16), and the mutation described in this report may have functional significance for the development of the malignant phenotype of this tumor.

## Materials and Methods

**Cell Lines.** The derivation of the 293-A24 stable transfectant of the 293 human kidney cell line expressing the HLA-A24 class I gene was previously described (9). The 293-A24 cells were maintained in DMEM containing 7.5% FBS, and melanoma (mel) cell lines maintained in RPMI containing 5% FBS. Melanoma-specific CTL were grown from TIL in AIM V (Life Technologies, Gaithersburg, MD) media containing 5% human serum plus 6,000 IU/ml IL2 for 30–60 d, as previously described (17). TIL 888 was grown from a tumor derived in 1989 from patient 888, and when administered to the autologous patient with IL2 resulted in the complete regression of multiple metastases. TIL 1290 was grown from a recurrent pelvic mass in patient 888 found three years later, and was administered in combination with TIL 888, resulting in regression of an additional pelvic tumor mass. TIL 1541 was grown from a small subcutaneous nodule isolated from patient 888 in 1995.

**cDNA Library Construction and Screening.** The cDNA library was constructed using 10  $\mu$ g of poly (A)<sup>+</sup> mRNA from the 1290 mel which had been doubly selected using the FastTrack polyA isolation kit (Invitrogen, San Diego, CA). The cDNA was synthesized using a random primer cDNA synthesis kit (Promega, Madison, WI), BstXI adaptors (Invitrogen) were ligated to the cDNA, and the cDNA was then cloned into pCDNA3 (Invitrogen) which had been digested with BstXI. Following the transformation of DH5a cells, the volume needed to obtain 50 bacterial transformants was calculated and used to inoculate 1 ml of Super Broth (Biofluids, Gaithersburg, MD) in a 96-well culture block (AGCT, Gaithersburg, MD) for 48 h. Plasmid DNA was then prepared using the 9600 Wizard system (Promega Corp., Madison, WI). Transfection of 293-A24 was carried out using 200 ng of plasmid DNA as previously described (15), 18–24 h later TIL were added, and 18–24 h later supernatants assayed for GM-CSF using a GM-CSF ELISA (R&D Sys., Inc., Minneapolis, MN).

**Sequencing and PCR Analysis.** DNA sequencing was conducted using the Sequenase 2.0 kit (United States Biochemical Corp., Cleveland, OH). A search of the GenBank database was carried out using the Blast sequence alignment algorithm (18). To carry out the reverse transcriptase (RT) PCR analysis, 5  $\mu$ g total cellular RNA was reverse transcribed using oligo-dT, and 1/10 of this reaction then used to carry a PCR. The PCR was carried out for 30 cycles at 95°C for 1 min, 60°C for 1 min and 72°C for 1 min. The PCR was carried out using the primers 60F (5'-AAG-GTCTGAGGAGCAGCTTC-3') and 727R (5'-TGGACCAT-AACTGCAGCCTT-3').

**Peptide Synthesis and Purification.** The peptides were synthesized on the Gilson AMS222 multiple peptide synthesizer using standard F-moc chemistry. Peptides were purified on a R2 reverse-phase HPLC column (PerSeptive Biosystems, Framingham, MA) as well as a C8 column (VYDAC, Hesperia, CA) using an aceto-

nitrile gradient in water with 0.05% trifluoroacetic acid, and were estimated to be >90% pure.

**Competitive Binding Assay.** A competitive peptide binding assay using live cells was carried out as previously described (19) using the HLA-A24 binding peptide AYIDNYNKF (20) and 888 EBV B cells, which express high levels of the HLA-A24 class I gene product. Briefly, cells were incubated overnight at 26°C in the presence of  $\beta$ 2-microglobulin, and the following day cells incubated with 10<sup>5</sup> cpm of the <sup>125</sup>I-labeled AYIDNYNKF peptide,  $\beta$ 2-microglobulin, and varying concentrations of unlabeled competitor peptides for 4 h at 20°C. Following the incubation period, cell-bound and unbound peptides were separated by centrifugation through a mixture of phthalate oils (dioctyl/dibutyl: 1:1.1), and the pellets counted in a gamma counter.

## Results

**Isolation of cDNA Clones.** A cDNA library was prepared from 1290 melanoma and pools containing approximately 50 cDNA clones were screened for reactivity with TIL 1290 by transient transfection into the recipient cell line 293-A24. This cell line was derived by stably transfecting the human kidney cell line 293 with the HLA-A24 class I gene derived from the 888 melanoma cell line and sorting cells which expressed this gene product at high levels (9). Following the screening of 800 pools containing a total of ~40,000 cDNAs two positive pools were isolated. Transfection of these pools into 293-A24 cells stimulated the release of 15 and 20 pg/ml of GM-CSF from TIL 1290, whereas transfection of the other pools resulted in the stimulation of less than 10 pg/ml of GM-CSF.

Individual clones were then isolated from the two positive pools, and positive clones from each of the two pools, designated 11a1 and 12g2, were identified which stimulated strong cytokine release from TIL 1290 following transfection into 293-A24 cells (Table 1). A comparable response was also seen using transfectants of p15, which had

**Table 1.** Antigen Recognition by TIL 1290 and 888

| Stimulator cell line | Transfected gene | HLA-A24 | GM-CSF Release* |         |
|----------------------|------------------|---------|-----------------|---------|
|                      |                  |         | TIL 1290        | TIL 888 |
|                      |                  |         | <i>pg/ml</i>    |         |
| 293-A24              | p15              | +       | 820             | 10      |
| 293-A24              | 12c2             | +       | 610             | 15      |
| 293-A24              | 11a1             | +       | 470             | 12      |
| 293-A24              | Tyrosinase       | +       | 30              | 1,700   |
| 293-A24              | None             | +       | 30              | 10      |
| 888 mel              | None             | +       | 3,500           | 4,100   |
| 624 mel              | None             | -       | 40              | <8      |
| None                 | None             | -       | 20              | 19      |

\*The release of cytokine from TIL 1290 and TIL 888 stimulated by 293-A24 transfectants or melanomas was measured following coincubation with the TILs for 24 h.

|                  |                |             |                         |            |                    |            |     |
|------------------|----------------|-------------|-------------------------|------------|--------------------|------------|-----|
|                  |                |             |                         |            |                    |            | 60  |
| $\beta$ -catenin | GGGGCAGCAG     | CGTTGGCCCG  | GCCCCGGGAG              | CGGAGAGCGA | GGGGAGGCCG         | AGACGGAGGA |     |
| 12g2             |                | -----       | -----                   |            | -----              | -----      |     |
| 11a1             |                |             |                         | GAACTGCTGA | GGTTGTAGG          | GGAT-----  |     |
|                  |                |             |                         |            |                    |            | 120 |
| $\beta$ -catenin | AGGTCTGAGG     | AGCAGCTTCA  | GTCCCCGCCG              | AGCCGCCACC | GCAGGTCGAG         | GACGGTCGGA |     |
| 12g2             | -----          | -----       | -----                   | -----      | -----              | -----      |     |
| 11a1             | -----          | -----       | -----                   | -----      | -----              | -----      |     |
|                  |                |             |                         |            |                    |            | 180 |
| $\beta$ -catenin | CTCCCGCGGC     | GGGAGGAGCC  | TGTTCCCCTG              | AGGGTATTTG | AAGTATACCA         | TACAACGTTT |     |
| 12g2             | -----          | -----       | -----                   | -----      | -----              | -----      |     |
| 11a1             | -----          | -----       | -----                   | -----      | -----              | -----      |     |
|                  |                |             |                         |            |                    |            | 240 |
| $\beta$ -catenin | TTGAAAATCC     | AGCGTGGACA  | M A T A T G C T A C T C | Q A D L    | M E L              | D M A      |     |
| 12g2             | -----          | -----       | -----                   | -----      | -----              | -----      |     |
| 11a1             | -----          | -----       | -----                   | -----      | -----              | -----      |     |
|                  |                |             |                         |            |                    |            | 300 |
| $\beta$ -catenin | M E P D        | R K A       | A V S                   | H W Q Q    | <u>Q S Y L D S</u> |            |     |
| 12g2             | TGGAACCAGA     | CAGAAAAGCG  | GCTGTTAGTC              | ACTGGCAGCA | ACAGTCTTAC         | CTGGACTCTG |     |
| 11a1             | -----          | -----       | -----                   | -----      | -----              | -----      |     |
|                  |                |             |                         |            |                    |            | 360 |
| $\beta$ -catenin | <u>G I H S</u> | G A T       | T T A                   | P S L S    | G K G              | N P E      |     |
| 12g2             | GAATCCATTC     | TGGTGCCTACT | ACCACAGCTC              | CTTCTCTGAG | TGGTAAAGGC         | AATCCTGAGG |     |
| 11a1             | -----          | -----       | -----                   | -----      | -----              | -----      |     |
|                  |                |             |                         |            |                    |            | 420 |
| $\beta$ -catenin | E E D V        | D T S       | Q V L                   | Y E W E    | Q G F              | S Q S      |     |
| 12g2             | AAGAGGATGT     | GGATACCTCC  | CAAGTCCTGT              | ATGAGTGGGA | ACAGGGATTT         | TCTCAGTCCT |     |
| 11a1             | -----          | -----       | -----                   | -----      | -----              | -----      |     |
|                  |                |             |                         |            |                    |            | 480 |
| $\beta$ -catenin | F T Q E        | Q V A       | D I D                   | G Q Y A    | M T R              | A Q R      |     |
| 12g2             | TCACTCAAGA     | ACAAGTAGCT  | GATATTGATG              | GACAGTATGC | AATGACTCGA         | GCTCAGAGGG |     |
| 11a1             | -----          | -----       | -----                   | -----      | -----              | -----      |     |
|                  |                |             |                         |            |                    |            | 540 |
| $\beta$ -catenin | V R A A        | M F P       | E T L                   | D E G M    | Q I P              | S T Q      |     |
| 12g2             | TACGAGCTGC     | TATGTTCCCT  | GAGACATTAG              | ATGAGGGCAT | GCAGATCCCA         | TCTACACAGT |     |
| 11a1             | -----          | -----       | -----                   | -----      | -----              | -----      |     |
|                  |                |             |                         |            |                    |            | 600 |
| $\beta$ -catenin | F D A A        | H P T       | N V Q                   | R L A E    | P S Q              | M L K      |     |
| 12g2             | TTGATGCTGC     | TCATCCCACT  | AATGTCCAGC              | GTTTGGCTGA | ACCATCACAG         | ATGCTGAAAC |     |
| 11a1             | -----          | -----       | -----                   | -----      | -----              | -----      |     |
|                  |                |             |                         |            |                    |            | 660 |
| $\beta$ -catenin | H A V V        | N L I       | N Y Q                   | D D A E    | L A T              | R A I      |     |
| 12g2             | ATGCAGTTGT     | AAACTTGATT  | AACTATCAAG              | ATGATGCAGA | ACTTGCCACA         | CGTGCAATCC |     |
|                  |                |             |                         |            |                    |            | 720 |
| $\beta$ -catenin | P E L T        | K L L       | N D E                   | D Q V V    | V N K              | A A V      |     |
| 12g2             | CTGAACTGAC     | AAAAGTCTA   | AATGACGAGG              | ACCAGGTGGT | GGTTAATAAG         | GCTGCAGTTA |     |
|                  |                |             |                         |            |                    |            | 780 |
| $\beta$ -catenin | M V H Q        | L S K       | K E A                   | S R H A    | I M R              | S P Q      |     |
| 12g2             | TGGTCCATCA     | GCTTCTTAAA  | AAGGAAGCTT              | CCAGACACGC | TATCATGCGT         | TCTCCTCAGA |     |
|                  |                |             |                         |            |                    |            | 840 |
| $\beta$ -catenin | M V S A        | I V R       | T M Q                   | N T N D    | V E T              | A R C      |     |
| 12g2             | TGGTGTCTGC     | TATTGTACGT  | ACCATGCAGA              | ATACAAATGA | TGTAGAAACA         | GCTCGTTGTA |     |
|                  |                |             |                         |            |                    |            | 890 |
| $\beta$ -catenin | T A G T        | L H N       | L S H                   | H R E G    | L L A              |            |     |
| 12g2             | CCGCTGGGAC     | CTTGCATAAC  | CTTCCCATC               | ATCGTGAGGG | CTTACTGGCC         |            |     |
|                  |                |             |                         |            |                    |            |     |

**Figure 1.** Sequence of the two  $\beta$ -catenin cDNA clones. The sequence of the two cDNA clones were aligned with the published  $\beta$ -catenin sequence, GenBank accession number Z19054 (29). The translated sequence is listed above the nucleotide sequence, with dashes indicating identity with the published sequence and periods indicating a gap in the 5' untranslated region of clone 12g2. The sequence of the peptide which represents the T cell epitope is underlined.

previously been shown to stimulate TIL 1290, but no response was seen with transfectants expressing tyrosinase. In contrast, TIL 888 responded to transfectants of tyrosinase but failed to respond to 293-A24 cells transfected with either the newly isolated clones or the p15 gene.

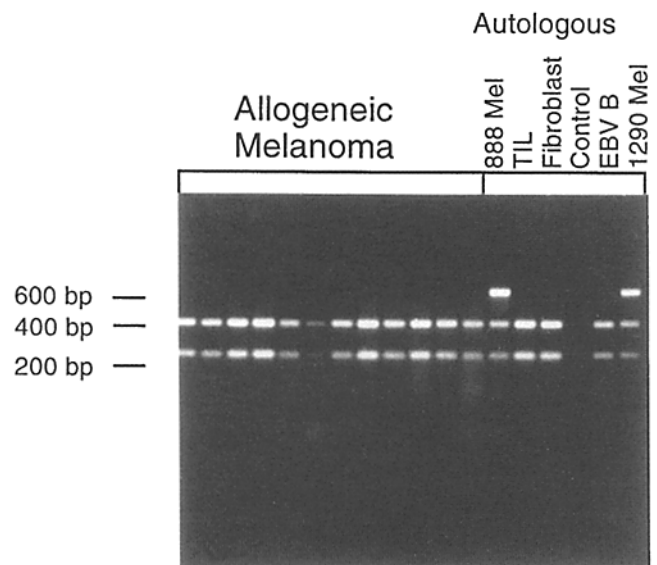
**Sequence Determination.** The sequences of the two positive clones were determined and a search of the GenBank database indicated that the sequences of both clones were nearly identical to that of the  $\beta$ -catenin gene (Fig. 1). The DNA sequence obtained for one of the cDNA clones, 12g2, corresponded to that found between residues 15 and 890 of the published human  $\beta$ -catenin sequence, but contained a deletion of 49 nucleotides within the 5' untranslated region. When RNA from the 1290 melanoma was reverse transcribed using oligo-dT and a PCR carried out using primers which flank this region the deletion was not observed, however, indicating that the deletion may represent a cloning artifact (data not shown). The sequence of the second clone, 11a1, corresponded to that of the region between residues 55 and 582 of the published  $\beta$ -catenin sequence. The 5' end of clone 11A1 contained 24 base pairs of unidentified sequence, which could either reflect alternative processing of this gene product or may again reflect a cloning artifact.

A comparison of the coding regions of the two cDNA clones with the published sequence of  $\beta$ -catenin demonstrated that a single nucleotide substitution of a T for a C residue was found in both of the cDNA clones at residue 310, resulting in a change of serine at position 37 to phenylalanine. To determine if this represented a mutation or an allelic polymorphism, RNA from autologous normal tissues as well as from the 888 and 1290 melanomas was reverse transcribed using oligo-dT, and a PCR carried out using two primers which flanked residue 310. The resulting 668 base pair product was then digested with XmnI, which should give rise to two products of 246 and 422 base pairs, in the case of the previously published sequence. The substitution found at residue 310 in the cDNA clones that were isolated, however, eliminated the XmnI restriction endonuclease site, and as a result the size of this product should not be altered following digestion with this enzyme. Digestion of the PCR products obtained using RNA from autologous fibroblasts, TIL or EBV cells with XmnI resulted in two bands of the expected size based on the published  $\beta$ -catenin sequence. Digestion of the PCR products derived from the 1290 melanoma RNA with XmnI resulted in a product of approximately 700 base pairs, corresponding to the undigested product, as well as two bands of about 250 and 400 base pairs (Fig. 2). The observation that the undigested product was found in the samples derived from the melanoma but not the normal tissues from patient 888 indicated that this represented a mutation and not an allelic polymorphism. In addition, the 1290 melanoma appeared to express the allelic  $\beta$ -catenin gene product which did not contain this mutation. The same pattern was observed for the 888 melanoma that had been isolated from the same patient three years earlier than the 1290 melanoma. When DNA sequencing reactions were carried out

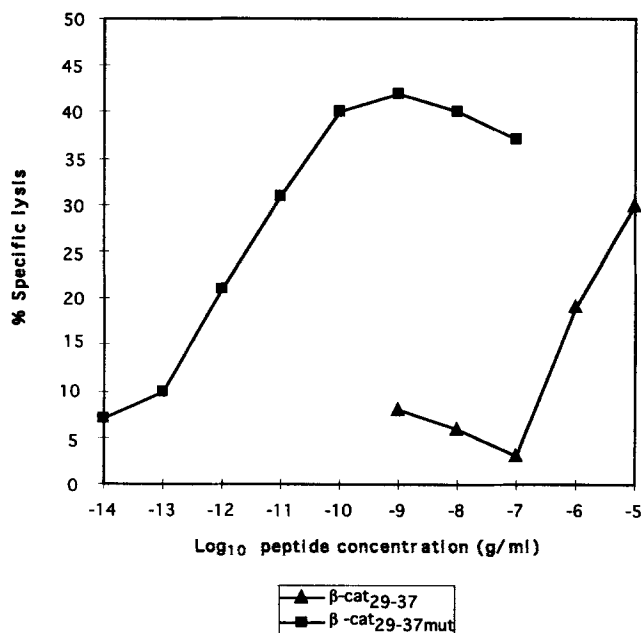
on the PCR products obtained from the 1290 and 888 melanoma, both a T and a C residue were observed at this position, confirming the results of the restriction enzyme digestion (data not shown). In addition, 12 allogeneic melanomas that were examined did not exhibit this mutation, indicating that this represented a relatively rare mutational event (Fig. 2).

**Recognition of Mutated  $\beta$ -Catenin Sequence.** An attempt was then made to identify the T cell epitope in  $\beta$ -catenin recognized by TIL 1290. The HLA-A24 binding motif was previously shown to consist of either a phenylalanine, leucine, isoleucine or tryptophan residue at the last position, and a tyrosine, phenylalanine, or methionine residue at position two (21). A peptide containing the amino acid 37 phenylalanine at position 9 would also contain a tyrosine at position two, which would conform to this motif. Therefore, the peptide corresponding to residues 29 to 37 of the mutant sequence, SYLDSGIHF ( $\beta$ -cat<sub>29-37</sub> mut), as well as the peptide corresponding to the normal allele, SYLDSGIHS, ( $\beta$ -cat<sub>29-37</sub>) were synthesized. When these peptides were tested for their ability to sensitize targets for lysis, the  $\beta$ -cat<sub>29-37</sub> mut peptide was found to sensitize target cells at a concentration of 1 pg/ml or higher, whereas concentrations of 1  $\mu$ g/ml or greater were required for sensitization of target cells with the normal  $\beta$ -cat<sub>29-37</sub> peptide (Fig. 3). Thus, TIL 1290 demonstrated 10<sup>6</sup>-fold better recognition of the mutant peptide than the normal peptide.

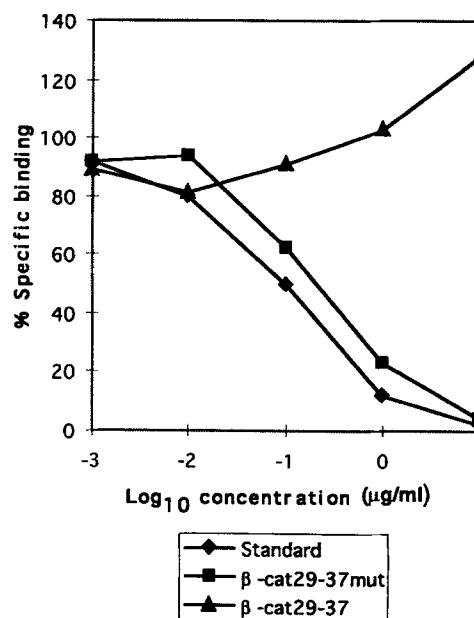
The effect of the mutation at position 37 on the affinity of this peptide for the HLA-A24 class I allele was then as-



**Figure 2.** Analysis of the expression of  $\beta$ -catenin gene products. 5  $\mu$ g of total RNA was reverse transcribed, and a PCR was then carried out using  $\frac{1}{10}$  of the product using primers 60F and 727R as described in the Materials and Methods. A restriction enzyme digest of the resulting 668 base pair product was then carried out with the enzyme XmnI and the products separated on a 1% agarose gel and stained with ethidium bromide. Digestion of the normal product resulted in two bands of 246 and 422 base pairs, whereas the mutant product, which lacked the XmnI restriction site, remained at 668 base pairs.



**Figure 3.** Titration of  $\beta$ -catenin peptides for recognition by TIL. 888 EBV B cells were incubated with the indicated concentrations of peptides and lysis by TIL1290 was measured in a 4-h  $^{51}\text{Cr}$  release assay at an E/T ratio of 40:1.



**Figure 4.** Competitive binding assay. The indicated peptides were diluted and tested for their ability to inhibit the binding of the labeled standard peptide AYIDNYNKF to 888 EBV B cells as described in the Materials and Methods. The average total and non-specifically bound cpm were 3,738 and 75 cpm, respectively. Non-specific binding was determined by incubating cells with the labeled peptide in the presence of 100  $\mu\text{g}/\text{ml}$  of the cold standard peptide.

sessed in a competitive binding assay. This assay was set up using 888 EBV B cells that expressed high levels of the HLA-A24 class I allele and an  $^{125}\text{I}$ -labeled peptide previously shown to bind HLA-A24 with high affinity (20). The result of this assay indicated that the concentration of the  $\beta$ -cat<sub>29-37mut</sub> peptide required for 50% inhibition of the binding of the labeled peptide was similar to that required for inhibition using the unlabeled standard peptide ( $\sim 100$  ng/ml for both peptides) (Fig. 4). In contrast, the normal  $\beta$ -cat<sub>29-37</sub> peptide did not significantly compete for binding at the highest concentration tested, 10  $\mu\text{g}/\text{ml}$ . This indicated that the mutation which occurred at position 37 in the  $\beta$ -catenin polypeptide was responsible for the generation a high affinity HLA-A24 binding peptide from a native sequence with little affinity for this class I allele.

A TIL line termed TIL 1541, which was recently derived from a recurrence in patient 888 that occurred three years after the derivation of TIL 1290. TIL 1541 was then tested for recognition of the mutant catenin gene product, as well as of tyrosinase and p15. Transfected targets expressing the mutant  $\beta$ -catenin gene product were recognized by TIL 1541, whereas transfectants expressing tyrosinase or p15 failed to stimulate any release of GM-CSF from this TIL (Table 2). In addition, TIL 1541 was found to lyse HLA-A24<sup>+</sup> 888 EBV B cells when incubated with as little as 0.1  $\mu\text{g}/\text{ml}$  of the  $\beta$ -cat<sub>29-37mut</sub> peptide, whereas concentrations of 1  $\mu\text{g}/\text{ml}$  or greater were required for recognition of cells pulsed with the normal  $\beta$ -cat<sub>29-37</sub> peptide (data not shown), similar to results found with TIL 1290.

In the cytokine assay in Table 2, strong stimulation of TIL 1290 was found with transfectants of the p15 gene,

whereas transfectants of the  $\beta$ -catenin gene product were only weakly recognized. This assay was carried out using a 51-d-old culture of TIL 1290, whereas the previous assay (Table 1) was carried out using a 38-d-old culture of TIL. The result of this as well as additional assays have demonstrated loss of reactivity with  $\beta$ -catenin between seven and eight weeks of culture of TIL 1290, whereas reactivity with p15 was maintained for several additional weeks (data not shown). The loss of reactivity may have resulted from the slower growth or more limited lifespan of  $\beta$ -catenin-reactive T cells relative to other T cells present in this culture.

The fresh un-cultured 1541 tumor was also analyzed for expression of the normal and mutated  $\beta$ -catenin sequence, in order to determine whether or not immunoselection of an antigen loss variant had occurred in vivo following treatment of the patient three years earlier with TIL 1290. The result of this analysis indicated that the 1541 tumor expressed the normal as well as the mutated  $\beta$ -catenin gene product (Fig. 5).

## Discussion

This report demonstrates that the mutated product of the  $\beta$ -catenin gene was recognized by the melanoma-specific TIL 1290. The mutation described in the  $\beta$ -catenin gene appeared to convert a peptide from a very low affinity binder to a very high affinity binder of the HLA-A24 class I gene product. Extremely high levels of the normal  $\beta$ -cat<sub>29-37</sub> peptide were shown to be required to sensitize target cells

**Table 2.** Antigen Recognition by TIL 888, 1290, and 1541

| Stimulator cell line | Transfected gene | HLA-A24 | GM-CSF Release |          |          |
|----------------------|------------------|---------|----------------|----------|----------|
|                      |                  |         | TIL 888        | TIL 1290 | TIL 1541 |
|                      |                  |         |                | pg/ml    |          |
| 293-A24              | p15              | +       | <8             | 1,360    | <8       |
| 293-A24              | $\beta$ -catenin | +       | <8             | 120      | 1,850    |
| 293-A24              | Tyrosinase       | +       | 2,400          | 11       | <8       |
| 293-A24              | None             | +       | <8             | 9        | <8       |
| 888 mel              | None             | +       | 5,000          | 930      | 3,100    |
| 397                  | None             | -       | <8             | <8       | <8       |
| none                 | None             | -       | <8             | 11       | <8       |

The cytokine release from TIL stimulated by 293-A24 transfectants following cocubation with TIL was measured after 24 h. The 12c2  $\beta$ -catenin cDNA construct was transfected in this assay.

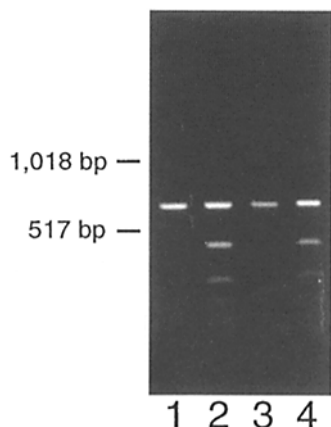
for lysis, and thus the non-mutated gene product may not be expressed at sufficient levels in normal cells to result in the processing and presentation of this peptide in association with HLA-A24. Two recent reports have demonstrated that melanoma-reactive T cells respond to products of mutated genes. In one, a mutant epitope derived from a previously unknown gene, termed MUM-1, was shown to be recognized by HLA-B44 class I restricted, melanoma-reactive T cells (13). A point mutation was found in this sequence that altered the amino acid sequence of the peptide epitope. The known primary anchor residues were not altered in this sequence, and the normal and mutant peptides bound equivalently to HLA-B44. Since the normal peptide was not recognized by melanoma-reactive T cells, this mutation appeared to have altered a T cell contact residue. In the second report, T cells were shown to recognize a mutated cyclin-dependent kinase 4 (CDK4) gene product (14). The mutation present in this gene product gave rise to a peptide with increased binding to HLA-A2.1, although a less dramatic difference was seen in the ability of the nor-

mal and mutant peptides from CDK4 to sensitize targets for lysis than was seen for the  $\beta$ -catenin peptides. The mutations in the CDK4 gene as well as in  $\beta$ -catenin involved a C-T transition at a dipyrimidine site, which may be associated with UV-induced damage in skin cancer (22).

The  $\beta$ -catenin gene product has been shown to be an intracellular protein involved in cell-cell adhesion. This protein binds to the cytoplasmic tail of cadherins, a family of cell surface proteins involved in cell-cell adhesion in a variety of cell types (16). The  $\beta$ -catenin product has been shown to bind to another intracellular protein,  $\alpha$ -catenin, and this complex appears to bind to intracellular actin microfilaments. We do not know whether the  $\beta$ -catenin mutation described in this report affects the function of this molecule. Frequent mutations of the epithelial cell adhesion molecule E-cadherin have been observed in certain cancers such as gastric carcinomas (23), and some studies suggest that disruption of the function of this gene product may play a role in cell metastasis (24). Mutations of the  $\beta$ -catenin gene (25) and  $\alpha$ -catenin gene (26) have been found in tumor cells and appeared to be associated with a loss of cell adhesiveness. Thus, the alteration of the normal function of  $\beta$ -catenin could be involved in development of the malignant phenotype, and it would be of interest to determine whether additional mutations in either the  $\beta$ -catenin gene or genes encoding other member of cell adhesion complexes are present in other melanomas. The product of the APC tumor suppressor gene has been shown to bind to  $\beta$ -catenin (27), and additional studies have demonstrated binding of  $\beta$ -catenin to the EGF receptor (28). These observations suggest that  $\beta$ -catenin may play multiple roles in intracellular signalling pathways, and provide other potential roles by which disruption of the function of this molecule could be involved in malignancy.

The TIL 1541 cell line, which was derived from a recent recurrence in patient 888, was also found to strongly recognize the  $\beta$ -cat<sub>29-37mut</sub> peptide, but failed to recognize tyrosinase or p15, two antigens previously shown to be recognized by TIL from this patient. It is difficult to assess the significance of this observation, since it is unclear whether the specificity of T cells isolated from TIL cultures reflects all of the specificity of T cells in vivo. The observation that the 888 tumor, as well as the recurrent 1290 and 1541 tumors, expressed the mutated  $\beta$ -catenin sequence indicates that reactivity of TIL with this epitope did not result in the selection of antigen loss variants in vivo, however, the level of expression of this epitope on the surface of tumor cells in vivo may be insufficient to allow efficient recognition by specific T cells. Expression of the mutated gene could also be involved with maintaining the malignant phenotype, as described above, resulting in selection of tumor cells that express this protein.

The demonstration in this report, as well as two others, that melanoma-reactive T cells can recognize the products of mutated genes raises the possibility that many patients with melanoma, as well as other tumors types, may contain T cells that react with mutant antigens. If certain proteins contain common mutations which give rise to T cell epi-



**Figure 5.** Expression of  $\beta$ -catenin sequences in 1290 and 1541 melanoma. Analysis of and digestion of RT-PCR products was carried out as described in the legend to Fig. 2. Lane 1, 1290 mel RT-PCR product uncut; lane 2, 1290 mel RT-PCR product digested with XmnI; lane 3, 1541 mel RT-PCR product uncut; lane 4, 1541 mel RT-PCR product digested with XmnI.

topes, they could potentially lead to the development of useful therapeutic reagents. These epitopes should also be truly tumor specific, which may help to avoid the development of potentially destructive auto-immune responses which could occur when patients are immunized with antigens expressed on normal tissues such as melanocytes and

retina. In addition, the high frequency of mutations found in tumors of many different histologies raises the possibility that T cells may be present in many of these patients which recognize mutated products, and that some relatively common mutations could serve as the targets of additional anti-tumor vaccines.

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